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13. ABSTRACT (Maximum 200 Words)

We cloned into a pVax expression vector the secreted and the truncated (no peptide leader sequence) versions of the human prostate acid phosphatase (H-PAP-T), the human prostate-specific antigen (H-PSA-T) and the rat analogue of the human PSMA (R-"PSMA"-S). A stable transfectant with H-PSMA and H-PSA of the AT3B1 cell line was obtained. The following plasmids were produced and purified under GLP-conditions using the Qiagen technology: H-PSMA-T, R-"PSMA"-T, H-PSA, H-PSA-T, H-PAP-T and R"PSMA"-S. Preliminary studies using the Copenhagen rat tumor prostate model showed uniform tumor development in rats that were injected subcutaneously with 100 000 AT3B-1PSMA,PSA cells. Using a commercially available transfection device from Amaxa, we could transfect differentiated dendritic cells with 20-40% efficiency. Such transfected dendritic cells stimulated in vitro autologous T cells to PSMA. T cells cytotoxicity was then tested against tumor cells or peptide-pulsed T2 target cells. Both H-PSMA-T DCs and S-PSMA DCs generated antigen-specific cytotoxic T cell responses. The immune response was restricted towards one of four PSMA derived epitopes when priming and boosting was performed with S-PSMA. In contrast, T-PSMA transfected DCs primed T cells towards several PSMA derived epitopes. Subsequent repeated boosting with transfected DCs restricted the immune response to a single immunodominant epitope.

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REPORT

Introduction:

Recent research indicates that loss of expression of certain antigens is a common phenomenon during cancer progression and, therefore, targeting a single, defined tumor antigen will be disadvantageous to inducing an immune response against a broad spectrum of potential targets. Furthermore, the generation of various peptides when the immunization is performed against different tissue-specific antigens will eliminate possible differences in the affinity for peptide binding of different MHC alleles thus increasing the probability of creating a universal tissue-specific vaccine for the highly polymorphic human population. For this reason, we speculated that a cancer vaccine targeting a multitude of tissue-specific antigens is more likely to raise an effective immune response. We have enlarged the target panel to include the prostate-specific membrane antigen (PSMA), the prostate acidic phosphatase (PAP) and the prostate-specific antigen (PSA). We plan to test the efficacy of immunization in an in vitro human model and the efficacy for tumor protection in a Copenhagen rat prostate tumor model.

Body of the report

1. Cloning of H-PAP, H-PSA and R-PSMA.

The human prostate specific antigen and the human prostate acidic phosphatase were cloned by RT-PCR of total RNA from LNCaP using specific primers.

The rat equivalent of the extracellular domain of the human prostate specific membrane antigen (R-PSMA) was cloned by RT-PCR of total RNA isolated from rat brain tissue. It has 85.66% homology with the human PSMA at the nucleotide level.

For the SH-PSMA and SR-PSMA plasmids, the extracellular portion of the human PSMA or its rat equivalent respectively, were cloned into pSecTag2 vectors (Invitrogen), which provided the murine Ig k-chain leader sequence. The inserts from the obtained clones were then sub-cloned into pVAX-1 expression vectors (Invitrogen).

A stable transfectant of the AT3B1 cell line, co-expressing the truncated H-PSMA and the secreted H-PSA, was obtained by standard technique with the commercially available reagent Fugene. The level of expression was tested and confirmed by Western blot for PSMA and PSA and by ELISA for the PSA.

2. Transfection of AT3B-1 cells

The rat AT3B-1 cell line expresses PAP. We have not been able to confirm expression at the protein level for PSMA and PSA in rat AT3-B1 cells. Since future immunotherapy experiments would use PSMA and PSA as targets for tumor development prevention, tumor cells needed to be stably transfected with both PSMA and PSA. A stable transfectant of the AT3B1 cell line, co-expressing the truncated H-PSMA and the secreted H-PSA, was obtained by standard technique with the commercially available reagent Fugene. Cells were initially transfected with PSA and selected with the

antibiotic G418 (neomycin). The level of expression was tested and confirmed by Western blot and ELISA. The cells were then transfected for HS-PSMA and selected with the antibiotics zeocin and neomycin.

3. GLP-level production and purification of the plasmid vectors

In order to proceed with the animal experiments as detailed in the Statement of Work of the original proposal, the plasmid vectors needed to be produced and purified under good laboratory practice (GLP) conditions. This was achieved using standard Qiagen technology. The following plasmids were produced and purified: H-PSMA-T, R-"PSMA"-T, H-PSA, H-PSA-T, H-PAP-T and R"PSMA"-S.

We performed the following DNA Quality Control:

- Endotoxin: Limulus amoebocyte lysate test: <100 E.U./mg DNA
- DNA homogenicity: >90% on agarose gel assay.
- RNA – no contamination on Agarose Gel and HPLC assays.
- E,Coli Genomic DNA contamination: <50 ug/mg DNA by Southern blot.
- Protein: Spectrophotometric scans between A220 and A320. Quantification is performed by use of the BCA protein test.
- Bioburden assay (for DNA of a final concentration not more than 1 mg/ml); 1 colonies following United States Pharmacopeia (USP 23) or Ph. Eur. Suppl 3,1998.

The structure of the DNA was monitored during production by restriction endonuclease analysis and following production by direct sequencing. It was not monitored prior to application.

Characteristic Specifications Assay

- Appearance: Clear, colorless solution on visual inspection
- Homogeneity of plasmid DNA >90% co-valently closed supercoiled circle using agarose gel
- Sterility Sterile Assay according to USP 23
- Presence of host DNA <5% (<0.05 mg/mg plasmid) by blot hybridization
- Presence of Proteins <1%(<0.01 mg/mg plasmid) by BCA colorimetric assay
- Presence of RNA and ssDNA: Not detectable on agarose gel and HPLC
- Presence of endotoxins <0.1 EU/μg plasmid using LAL test according to USP 23
- Plasmid identity: exclusive presence of target plasmid by restriction digest, sequencing

4. Development of the Copenhagen rat prostate tumor model

Preliminary studies using the Copenhagen rat tumor prostate model showed uniform tumor development in rats that were injected subcutaneously with 100 000 AT3B-1^{PSMA,PSA} cells. Healthy, Copenhagen 2331 male retired rat breeders were injected

sub-cutaneously in the right flank with $1x10^5$ tumor cells from the AT3B-$1^{PSMA,PSA}$ rat prostate carcinoma cell line. Injections were performed using 25-ga needles. Tumor size was evaluated every other day by measuring two perpendicular diameters by a caliper. The animals were observed twice daily for morbidity and mortality, and once daily for clinical signs of toxicity (cage side). A more thorough examination was performed at weekly intervals. Animals were monitored for weight on weekly intervals throughout the whole duration of the study. Two weeks after tumor inoculation all rats were sacrificed, tumor were excised and measured. Necropsy was performed, tumor and sera was collected and liver and lungs were inspected macroscopically for metastases.

Good size (~2 cm3) subcutaneous tumors were established two weeks following injection. Tumors did not invade muscle and there were no skin ulcerations.

5. In vitro transfection of human dendritic cells with PSMA

We next needed to test the efficacy of transfection of human dendritic cells with PSA and PAP and the ability of such transfected Using a commercially available transfection device from Amaxa, we could transfect differentiated dendritic cells with 20-40% efficiency. Such transfected dendritic cells stimulated in vitro autologous T cells to PSMA. T cells cytotoxicity was then tested against tumor cells or peptide-pulsed T2 target cells. Both H-PSMA-T DCs and S-PSMA DCs generated antigen-specific cytotoxic T cell responses. The immune response was restricted towards one of four PSMA derived epitopes when priming and boosting was performed with S-PSMA. In contrast, T-PSMA transfected DCs primed T cells towards several PSMA derived epitopes. Subsequent repeated boosting with transfected DCs restricted the immune response to a single immunodominant epitope. The results have been incorporated in a manuscript which has been submitted for publication in Cancer Gene Therapy [1].

Key Research Accomplishments

- Cloning of H-PAP, H-PSA and R-PSMA.
- Design and production of constructs for H-PAP and H-PSA that have no leader sequence and whose expression products are cytosolically retained and degraded in the proteasome (H-PSA-T and H-PAP-T)
- Design and production of constructs that lead to secreted H-PSMA (SHPSMA) and secreted R-PSMA (SR-PSMA).
- Production and purification of GLP-grade gene-based vaccines for use in the Copenhagen rat prostate tumor protection model (H-PSMA-T, R-"PSMA"-T, H-PSA, H-PSA-T, H-PAP-T and R"PSMA"-S)
- Obtain a stable transfectant of the AT3-B1 cell line co-expressing the human PSA and the human PSMA ($AT3B\text{-}1^{PSA,PSMA}$)
- Develop a tumor development model in a Copenhagen rat with the stably-transfected tumor cells $AT3B\text{-}1^{PSA,PSMA}$
- Study the efficacy of human dendritic cells transfected with the H-PSMA plasmid to stimulate autologous T cells in vitro. Test their ability to lyse target tumor cells or HLA-identical cells that have been pulsed with PSMA plasmids [1].

Reportable Outcomes

1. Manuscript: Mincheff M, Zoubak S, Altankova I, Tchakarov S, Makogonenko Y, Botev C, Ignatova I, Dimitrov R, Madarzhieva K, Hammett M, Pomakov Y, Meryman H, Lisstchkov T. Human Dendritic Cells Genetically Engineered to Express Cytosolically-Retained Fragment of Prostate-Specific Membrane Antigen Prime Cytotoxic T Cell Responses to Multiple Epitopes. Cancer Gene Therapy (submitted for publication).
2. Gene-based vaccines for immunotherapy of cancer:
    - H-PSA – a plasmid vector that contains an expression cassette for the human PSA
    - H-PSA-T – a plasmid vector that contains an expression cassette for the human PSA with no signal peptide
    - H-PAP – a plasmid vector that contains an expression cassette for the human PAP
    - H-PAP-T – a plasmid vector that contains an expression cassette for the human PAP with no signal peptide
    - R-PSMA – a plasmid vector that contains an expression cassette for the mouse analogue of the extracellular domain of the human PSMA
    - SR-PSMA – a plasmid vector that contains an expression cassette for the mouse analogue of the extracellular domain of the human PSMA with a signal peptide
    - SH-PSMA – a plasmid vector that contains an expression cassette for the extracellular domain of the human PSMA with a signal peptide
3. AT3B-1^{PSMA,PSA} – a rat AT3B-1 cell line that secretes the extracellular domain of the human PSMA and the human PSA

Conclusions

The research has progressed according to the approved Statement of Work. There have been no problems so far connected with the experimental design except for the fact that PSMA expression in the AT3B-1 cell line has been detected only at the mRNA but not protein level. For this reason we have selected to transfect the cell line, in addition to PSA, with PSMA. Consequently, all tumor protection studies will be performed with the AT3B-1^{PSA,PSMA} cell line.

References

1. Mincheff M, Zoubak S, Altankova I, Tchakarov S, Makogonenko Y, Botev C, Ignatova I, Dimitrov R, Madarzhieva K, Hammett M, Pomakov Y, Meryman H, Lisstchkov T. Human Dendritic Cells Genetically Engineered to Express Cytosolically-Retained Fragment of Prostate-Specific Membrane Antigen Prime Cytotoxic T Cell Responses to Multiple Epitopes. Cancer Gene Therapy (submitted for publication).

Appendices

1. Manuscript submitted for publication:
Mincheff M, Zoubak S, Altankova I, Tchakarov S, Makogonenko Y, Botev C, Ignatova I, Dimitrov R, Madarzhieva K, Hammett M, Pomakov Y, Meryman H, Lisstchkov T. Human Dendritic Cells Genetically Engineered to Express Cytosolically-Retained Fragment of Prostate-Specific Membrane Antigen Prime Cytotoxic T Cell Responses to Multiple Epitopes. Cancer Gene Therapy (submitted for publication).

Cancer Gene Therapy, 2003 (submitted)

Human Dendritic Cells Genetically Engineered to Express Cytosolically-Retained Fragment of Prostate-Specific Membrane Antigen Prime Cytotoxic T Cell Responses to Multiple Epitopes

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ABSTRACT

The ability of two plasmid DNA vaccines to stimulate lymphocytes from normal human donors and to generate antigen-specific responses is demonstrated. The first vaccine (truncated; tPSMA) encodes for only the extracellular domain of PSMA. The product, expressed following transfection with this vector, is retained in the cytosol and degraded by the proteasomes. For the "secreted" (sPMSA) vaccine, a signal peptide sequence is added to the expression cassette and the expressed protein is glycosylated and directed to the secretory pathway. Monocyte-derived dendritic cells (DCs) are transiently transfected with either sPSMA or tPSMA plasmids. The DCs are then used to activate autologous lymphocytes in an in vitro model of DNA vaccination. Lymphocytes are boosted following priming with transfected DCs or with peptide-pulsed monocytes. Their reactivity is tested against tumor cells or peptide-pulsed T2 target cells. Both tPSMA DCs and sPSMA DCs cells generate antigen-specific cytotoxic T cell responses. The immune response is restricted towards one of the four PSMA derived epitopes when priming and boosting is performed with sPSMA. In contrast, tPSMA transfected DCs prime T cells towards several PSMA derived epitopes. Subsequent repeated boosting with transfected DCs, however, restricts the immune response to a single epitope due to immunodominance.

INTRODUCTION

Immunotherapy of prostate cancer could be a safe, non-invasive, relatively inexpensive procedure that can avoid bowel and bladder injury and impotence often resulting from surgical, cryosurgical or radiation therapy. Several groups have recently reported on the safety of DNA vaccines for immunizations against tumor antigens.[1-3] A possible target for prostate cancer immunotherapy is the prostate specific membrane antigen (PSMA), also known as glutamate carboxypeptidase II (GCPII).[4-8] PSMA expression is normally restricted to the prostate gland, brain tissue, jejunum and proximal kidney tubules.[9-11] Its expression is increased nearly 10-fold in prostate cancer cells and is also found in tumor but not normal neovasculature.[7,12]

The main effectors in anti-tumor immunity are $CD8^+$ cytotoxic T cells that recognize tumor-associated antigen-derived peptides in association with major histocompatibility complex (MHC) class I molecules.[13-15] Gene-based vaccination in its current mode of application is effective in breaking tolerance to a self-antigen, but the response is narrow and is restricted to few of the potential epitopes. This presents a problem in vaccinology since loss of an MHC haplotype that participates in the conformation of the T cell antigen, or point mutation in the recognized sequence would result in ineffective immune surveillance.[16-18] New vaccines and/or new methods of immunizations need to be developed for those instances. These hopefully will raise responses to subdominant determinants so that the selection of tumor escape variants that fail to express immunodominant epitopes will be prevented.[16]

Numerous factors combine to establish an immunodominance hierarchy,[19] among them the ineffective generation and transport of sub-dominant epitopes by antigen-presenting cells (APCs). Since proteasomal degradation is the main source of antigenic fragments destined for MHC presentation,[14] we speculated that purposeful cytosolic retention of newly synthesized tumor-associated antigens in genetically manipulated antigen presenting cells may increase both the quantity and the diversity of such fragments. PSMA is a type II transmembrane glycoprotein which is comprised of 750 amino acids.[20,21] It lacks a signal peptide sequence and we speculated that elimination of sequences for its transmembrane region might impede the translocation of the encoded product to the endoplasmic reticulum. Such product should not be N-glycosylated, should be retained in the cytosol and rapidly degraded in the proteasome. In theory, DC transfected with such "truncated" sequences, may have an advantage of presenting "subdominant" antigenic determinants that otherwise may not be generated at sufficient density to prime antigen-specific cytotoxic T cell responses. The following experiments were designed to test this hypothesis in an in vitro immunization system with human cells.

MATERIALS AND METHODS

All human cellular material used in these experiments was obtained following informed consent through protocols approved by the local Committee for Bioethics (Bulgaria) or the Investigational Review Board (IRB) at George Washington University Medical Center in Washington, DC.

tPSMA and sPSMA plasmids construction

The cDNA encoding the extracellular portion (AA 44-750) of the human PSMA (XC-PSMA) was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) after RT-PCR of total mRNA from the human prostate cancer cell line LNCaP (CRL 1740, ATCC). The forward primer introduced a NotI cloning site and a Kozak sequence with a Met codon (GCCACCATG) into the 5'-end of XC-PSMA. The tPSMA plasmid for the transfection experiments was obtained by NotI-XhoI sub-cloning of XC-PSMA into a pVAX1 mammalian expression vector (Invitrogen, Carlsbad, CA). A secretable variant of the tPSMA plasmid – the sPSMA plasmid - was obtained by fusion of the XC-PSMA with a murine Ig k-chain leader sequence. The insert from tPSMA plasmid was sub-cloned by BamHI-XhoI into the mammalian expression vector pSecTag2A (Invitrogen, Carlsbad, CA) providing the murine Igk-chain leader sequence (clone 96). The single SfiI cloning site of the vector was used to fuse the XC-PSMA in-frame with the leader. The 5'-portion of the XC-PSMA between start Met and a single HpaI site was re-amplified in order to introduce SfiI site (Met codon was not included). The SfiI-HpaI fragment of clone 96 was replaced with the PCR product pre-digested with the same restriction endonucleases and the NruI-XhoI fragment from obtained construct was moved to the pVAX1 vector. The inserts in both constructs are under the regulation of a human cytomegalovirus (CMV) immediate-early promoter/enhancer and a bovine growth hormone polyadenylation signal. The plasmid DNA specifications include endotoxin content below 0.1 EU per microgram of DNA; lack of detectable amounts of bacterial RNA, genomic DNA or ssDNA as determined by agarose-gel electrophoresis; less than 10 microgram of protein per 1 mg of plasmid DNA as determined by colorimetric assay (Bio-Rad, Hercules, CA).

COS-1 transfection

Expression of PSMA constructs was performed in Cos-1 cells (ATCC). Monolayers were transfected with FuGENE 6 transfection reagent (Roche) and assayed for PSMA production by Western blot. Cos-1 cells were seeded in 6-well tissue culture plates (Nunc, Denmark) at $1.5x10^5$ cells per well and grown to 50-70% confluence in DMEM supplemented with 25 mM HEPES (pH 7.5), 1 mM sodium pyruvate, 3.7 g/L sodium bicarbonate, 100 µg/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B and 10% (v/v) of heat inactivated fetal bovine serum. Cos-1 cells were transfected with 1.5 µg of plasmid DNA pre-condensed with 4.5 µl of FuGENE 6 reagent in serum-free DMEM for 30 min at room temperature. Cells were then grown for 72 h in supplemented DMEM and then harvested.

In the proteasome inhibition studies, lactacystin (Sigma) was added to the culture media (final concentration 10 µM) 24 h before harvesting.

Cells were harvested by gentle scraping after double wash with 2 ml of cold PBS and by adding of 0.25 ml of cold lysis buffer (0.5 M NaCl, 1% triton X-100, 0.2% Tween 20, 50 mM HEPES, pH 7.0) to each well. Lysates were transferred to Eppendorf tubes and homogenized by repeated pipetting on ice.

For detection of secreted PSMA, the serum containing DMEM was removed 48 h after transfection, the cells were washed twice with 2 ml of PBS, serum free DMEM (2 ml per well) was added and cells were incubated for additional 24 h in the 6-well plates. After collection of the medium, the cell debris was removed by centrifugation (35,000 g, 20 min) and supernatants were concentrated with Centricon-50 centrifuge filtering device (Millipore) and stored at -30°C until further use.

Deglycosylation of PSMA

The deglycosylation of PSMA proteins was carried out by treatment of 25 mg protein samples with 5 units of glycopeptidase F from Chryseobacterium Meningosepticum (Sigma) in 50 mM phosphate buffer (pH 7.5) containing 0.1% (w/v) SDS, 50 mM â-mercaptoethanol, 0.75% (v/v) Triton X-100 for 4 h at 37°C.[22]

An extracellular portion of the human glutamate carboxypeptidase II (amino acids 44-750) in Drosophila Schneider's cells, purified to homogeneity [23], was provided to us by Dr. Jan Konvalinka, Institute of Organic Chemistry and Biochemistry, Czech Academy of Science, Prague, The Czech Republic.

Electrophoresis and immunoblotting

Immunoblotting and electrophoretic assays of cell lysates and culture supernatants were performed using NuPage BioTris electrophoretic system (Invitrogen). Protein samples in loading buffer were heated at 70°C for 10 min and loaded on 10% Bis-Tris gels. After electrophoresis and electro-transfer, the nitrocellulose membrane was blocked with 1% casein in TBS/T for 40 min. The membranes were probed with poly- or monoclonal anti-PSMA Abs (see below) for 1 h at RT. Detection was performed using goat anti-rabbit or anti-mouse IgG-HRP conjugates (Sigma) and visualized with WestPico Super Signal Chemo luminescent Substrate (Pierce) in accordance with the manufacturer's recommendations.

Anti-human PSMA monoclonal antibodies Y-PSMA1 and Y-PSMA2 were obtained from Yes Biotech Laboratories Ltd (Mississauga, Ontario, Canada).

Cell culture and generation of monocytes-derived dendritic cells

Cell cultures from peripheral blood mononuclear cells were maintained in research grade serum-free AIM-V medium (Invitrogen, Carlsbad, CA). The human prostate cancer cell line LNCaP was purchased from ATCC and was maintained in RPMI supplemented with 10% FCS (Life Technologies Inc., Rockville, MD), 2 mM L-glutamine, 50 units/ml penicillin and 50 mg/ml streptomycin. The human T2 cell line is transporter associated with antigen processing (TAP) deficient, resulting in inefficient loading of leukocyte antigen class I molecules with endogenous peptides.[24] As a consequence, the HLA-A0201 molecules of T2 cells can be efficiently loaded with exogenous peptides. The T2 cell line was purchased from ATCC and maintained in IMDM supplemented with 20% FBS (Life Technologies Inc., Rockville, MD).

For T cell stimulation, the leukocyte fraction was collected by leukapheresis and mononuclear cells were separated on a density gradient. Cells were resuspended in serum-free AIM-V medium at $2x10^7$/ml and incubated for 2 hrs in a humidified incubator at 37°C. The non-adherent T-cell enriched fraction and part of the adherent cells were harvested and frozen for future use.

The rest of the adherent cells were differentiated into DCs by culture in serum-free AIM-V medium supplemented with IL-4 (PeproTech, Rocky Hill, NJ) and GM-CSF (Oncology

Supplies Inc. (Dothan, AL) for 6 days. The non-adherent cells were harvested and used for transfection. Transfection was performed using the Nucleofector™ device and corresponding transfection kit (Amaxa GmbH, Cologne, Germany. The efficiency of Nucleofector™ transfection of human DCs was 32±8.8% (n=5) as estimated by control transfection with green fluorescence protein (GFP). Following transfection, dendritic cells were resuspended in serum-free AIM-V medium and matured with TNF-α (Becton Dickinson Inc., Bedford, MA) at 37°C for 24 hours. Following maturation, the DCs were resuspended in AIM-V medium at $1x10^5$ cells/ml.

In Vitro Generation of CTL Responses

For T cell stimulation, the T-cell enriched fraction was thawed, washed, resuspended in AIM-V medium and added to the DC suspension so that the final concentration of the T–cell enriched fraction was $1x10^6$ cells/ml. The cell suspension was then distributed into 24-wells plates (1ml/well) and cultured at 37°C. Three days later, the medium was replaced with serum free AIV-M medium supplemented with human IL-2 (20 U/ml) and human IL-7 (10 U/ml) (PeproTech, Rocky Hill, NJ). Cells were additionally stimulated with autologous PSMA-transfected DCs (stimulator:effector ratio of 1:10) or with peptide-pulsed autologous monocytes (stimulator:effector ratio of 1:1) twice, 8 days apart.

For pulsing with peptides, monocytes were resuspended at 10^6/ml in serum-free RPMI-1640 medium supplemented with L-glutamine and penicillin/streptomycin. Peptide was added to a final concentration of 0.05 mg/ml and the cells were incubated for 4 hours at 37°C in a controlled CO_2 humidified incubator. The cells were then washed twice with serum-free medium, irradiated (1500 rads) and resuspended in IL-2 and IL-7 containing medium.

For cytotoxicity assays, target T2 cells were pulsed for 6 hours with 0.05 mg/ml peptide and 1ì Ci/well ^{3}H-thymidine (ICN Biomedical Inc., Irvine, CA).

After 20 days of culture, effector T cells were harvested without further separation for micro-cytotoxicity assays. The cells were analyzed by flow cytometry and 83±10% of them were $CD3^+$ and ~ 45% or ~60% of them were $CD3^+CD8^+$ when primed with sPSMA DCs or tPSMA DCs respectively (data not shown).

For CTLA-4 inhibition experiments, Fab-fragments, prepared (see below) from anti-CD152 (HB-12319, ATCC) monoclonal antibody, were added (0.02 mg/ml final concentration) to the T cells at the initiation of culture or 6, 10 or 18 hours after that. Cultures were incubated for 3 additional days and the cells were washed and re-suspended in IL-2 and IL-7 containing AIV-M medium.

Fab-fragment preparation of anti-CD152 monoclonal antibodies

Purified IgG (2 mg/ml in PBS) was cleaved with papain (Sigma) at a ratio 1:25 (w/w) of papain to IgG in the presence of 0.01 mM L-cystein at 37°C for 5.5 h. The reaction was stopped by the addition of N-ethylmaleimide to a final concentration of 30 mM. The Fab fragment was isolated by gel filtration with a Superdex 75 column (Pharmacia) followed by ion exchange column (ResourceQ, Pharmacia) chromatography. The buffer for the eluted protein was changed to 10 mM Tris-HCl (pH 7.4) by gel filtration and the pooled protein was concentrated to 15 mg/ml with centricon-10 (Millipore). The protein was tested for purity at each step by SDS-PAGE under reduced and non-reduced conditions.

Cytotoxicity testing

Cytotoxicity against LNCaP cells or against peptide pulsed T2 cells (both HLA-A2-positive) was tested after 20 days of culture and compared to a control cell line that did not express PSMA. Cytotoxicity was tested using the JAM test.[25] Briefly, target or control cells were grown overnight with ^{3}H-thymidine, then washed, resuspended in complete RPMI-1640 medium and used in 4-hour cytotoxicity test. The killing was detected as a fall in counts per minute in cell samples undergoing apoptosis due to DNA fragmentation. All of the E:T ratios were tested in triplicate. Spontaneous cytotoxicity was determined in medium alone without effector cells.

Unlabelled K562 cells (no MHC expression, sensitive to natural killer cell-mediated lysis) were included at 50 x the target cell number to inhibit nonspecific lysis. Control experiments involved the Malme M3 melanoma cell line, which is also HLA-A2 positive.

Cell Lines

The human LNCaP (CRL-1740), T2 (CRL-1992), K562 and Malme 3M (HTB-64) cell lines were purchased from ATCC and were maintained according to ATCC instructions.

Flow cytometry analysis and antibodies used

Antibodies used to phenotype the cells were anti-CD1a, anti-HLA-DR-PE, anti-CD80-PE, anti-CD86-PE, anti-CD83-FITC, anti-CD54-PE, anti-HLA-ABC-FITC, anti-CD14-FITC, anti-CD8-PE, anti-CD4-PE, anti-CD69-FITC (PharMingen, San Diego, CA), and anti-CD3-PerCP (Becton Dickinson, San Jose, CA). For HLA-A2 Typing, aliquots of peripheral blood mononuclear cells (PBMC) of healthy donors were tested with the FITC-labeled anti-HLA-A2 antibody BB7.2 (Becton Dickinson). For staining, 10^{5} cells were suspended in 100 µl of PBS 1% BSA and were incubated with 10 µl of the antibodies for 20 min on ice. Flow cytometry analysis was performed on a FACS-Calibur (Becton Dickinson).

Cytokines and ^{3}H-thymidine

GM-CSF was purchased from Oncology Supplies Inc. (Dothan, AL), IL-4 and IL-7 from PeproTech Inc. (Rocky Hill, NJ), IL-2 and TNF-α from Becton Dickinson Inc. (Bedford, MA).

^{3}H-thymidine was purchased from ICN Biomedical Inc (Irvine, CA).

Statistics and Epitope Binding Predictions

Analysis of cytotoxicity data were performed using two-tailed Student's t tests assuming equal variance. We used the predictive algorithm from the Bioinformatics and Molecular Analysis Section of the NIH ("BIMAS") that was developed by Parker et al[26], ranking potential MHC binders according to the predictive one-half-time disassociation of peptide/MHC complexes.

Peptide synthesis and purification

Peptides were custom synthesized and purified by Sigma Genosys (The Woodlands, Texas).

RESULTS

Selection of HLA-A2-binding PSMA peptides

The aminoacid sequence of the extracellular domain of PSMA was analyzed for the existence of 9-aminoacid peptides predicted to bind to HLA-A0201, the most common human MHC class I allele. Using the computer-based algorithm (http://bimas.cit.nih.gov/) four 9-mer peptides that contains binding motifs for the HLA-A0201 class I molecule were identified (table 1).

Table 1. HLA-A0201-restricted PSMA-derived peptides

Rank	Position at PSMA molecule*	Peptide Sequence	Score**
1	663	MMNDQLMFL	1360
2	711	ALFDIESKV	1055
3	668	LMFLERAFI	261
4	707	GIYDALFDI	251

* positions determined using the numbering from Israeli et al[4].

** score corresponds to the estimated half-time of dissociation of complexes containing the peptide at 37°C at pH 6.5

COS-1 cells transfected with tPSMA or sPSMA plasmids express the encoded product

Following transfection with sPSMA plasmid, the encoded product is N-glycosylated (fig.1) and could be detected intra- and extracellularly – i.e. is secreted (data not shown). The product expressed following transfection with the tPSMA plasmid is not glycosylated, but is retained in the cytosol and rapidly degraded in the proteasome. It could be detected following proteasomal inhibition with lactacystin (fig.2).

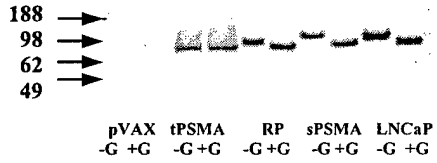

Fig.1. COS-1 cells were transfected with empty (pVAX), tPSMA or sPSMA plasmids, grown for 72 hours and the cell lysates were analyzed on Western blot with (+G) or without (-G) prior treatment with glycopeptidase F. Unlike the product obtained following transfection with tPSMA, the product obtained following transfection with sPSMA is N-glycosylated. Bands from recombinant PSMA or from cell lysates from LNCaP cells are shown for comparison. Legend – pVAX, tPSMA and sPSMA – lysates from COS-1 cells transfected with the respected plasmids; RP – recombinant PSMA expressed in insect cells, LNCaP – cell lysate form LNCaP.

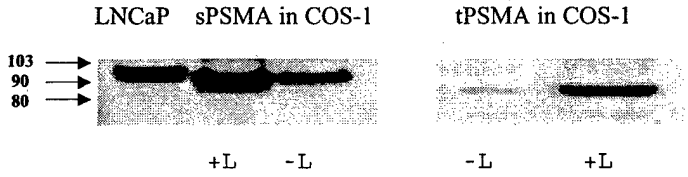

Fig 2. COS-1 cells were transfected with the tPSMA plasmids and the cells were grown for 72 hours, the last 24 hours in the presence of lactacystin (10 μM). Unlike the product expressed following transfection with sPSMA (left panel), most of the product expressed following transfection with tPSMA (right panel) is degraded in the proteasome and could be detected following proteasomal inhibition. Legend –LNCaP- LNCaP cell lysate; +/- L – in the presence (+) or absence (-) of the proteasomal inhibitor lactacystin.

Generation of mature DCs from peripheral blood monocytes

Immature DC were generated from PBMC after 6 days of culture in GM-CSF- and IL-4-conditioned medium. DC appeared as non-adherent cells with the typical DC morphology and high expression of CD1a (80-90%). Treatment of tPSMA- and sPSMA-transfected DCs with TNF-á triggers a coordinate series of phenotypic changes, resulting in an up-regulation of co-stimulatory molecules (CD80, CD86, CD40) and HLA class II antigens (fig.3).

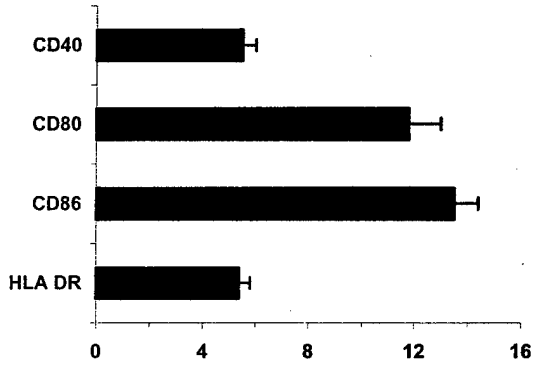

Fig.3. Change in cell surface antigen expression in PSMA-transfected DCs following maturation with TNF-α. Since surface antigens shows a heterogeneous baseline expression, the up-regulation of those molecules is presented as a ratio of mean fluorescent intensity (MFI activated/MFI resting). Data from 5 experiments with different donors are shown.

Both tPSMA DCs and sPSMA DCs prime and support development of T cells that are cytotoxic against LNCaP cells

The T cell enriched fraction from each leukapheresis was primed and then boosted twice, at 8-day intervals with autologous PSMA-transfected DCs. Their cytotoxicity was then tested against LNCaP cells or control Malme M3 melanoma cells. Both tPSMA DCs and sPSMA DCs primed and supported development of T cells that are cytotoxic against LNCaP cells (fig.4).

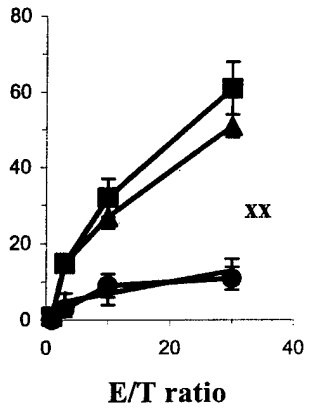

Fig. 4. Generation of CTL by tPSMA DCs (squares) or sPSMA DCs (triangles). Non-adherent (T cell-enriched) HLA A2 (+) peripheral blood mononuclear cells were primed and then boosted twice at 8-day intervals with autologous monocytes-derived dendritic cells that were transiently transfected with either tPSMA plasmid or sPSMA plasmid. The medium was changed, initially 72 hours following priming, and then during boosting. The cells were grown in IL-2 and IL-7 medium for 20 days and the specific cytotoxicity tested against LNCaP. Each point represents the mean and SD of triplicate experiments. Significant differences ($p<0.01$) in specific cytotoxicity at the 30:1 E/T ratio are indicated (xx). Control experiments involved priming and boosting of the T-cell enriched fraction with DCs transfected with empty plasmid (diamonds) or testing of cytotoxicity against the Malme-3M melanoma cell line (circles).

tPSMA DCs, but not sPSMA DCs prime T cells that are reactive to sub-dominant PSMA epitopes

Since proteasomal degradation is the main source of antigenic fragments destined for MHC class I presentation,[14] and since purposeful cytosolic retention of non-glycosylated and misfolded newly synthesized tumor-associated antigens enhances such degradation (fig.2), we reasoned that tPSMA transfected DCs will have a greater potential to prime T cells specific for sub-dominant PSMA epitopes. To check this hypothesis, we primed the T cell enriched fraction from each leukapheresis with mature tPSMA DCs or sPSMA DCs and boosted them twice, at 8-day intervals, with autologous monocytes pulsed with one of several PSMA derived peptides (table 1). Twenty days after priming, cytotoxicity was tested against T2 cells pulsed with the same peptide used for boosting. Unlike the dendritic cells pulsed with the sPSMA plasmid, the tPSMA DCs prime T cells to all four PSMA-derived peptides in four of the five donors (fig.5).

CTLA-4 inhibition during sPSMA DC-priming leads to stimulation of T cells reactive to sub-dominant epitopes

Lack of responses to the subdominant PSMA epitopes when DCs are transfected with sPSMA may result from complete lack of such epitopes on the membrane of the APCs. Alternatively, the epitopes may be generated but factors other than T-cell receptor (TCR) signaling such as the CTLA-4/B7 pathway, could be contributing to the ineffective proliferation of T cell to subdominant epitopes.

Currently, there is a general consensus that effective activation of naive T cells requires two signals: one dependent on the engagement of the TCR by peptide-MHC complexes, and the second co-stimulatory signal that is provided by interactions between cell surface molecules on the T cell and the APC.[27] Numerous studies have indicated that the CD28 molecule, expressed on T cells, provides a potent co-stimulatory signal following engagement with its ligands, B7-1 and B7-2.[28] The co-stimulatory function of CD28 is counterbalanced by the existence of a

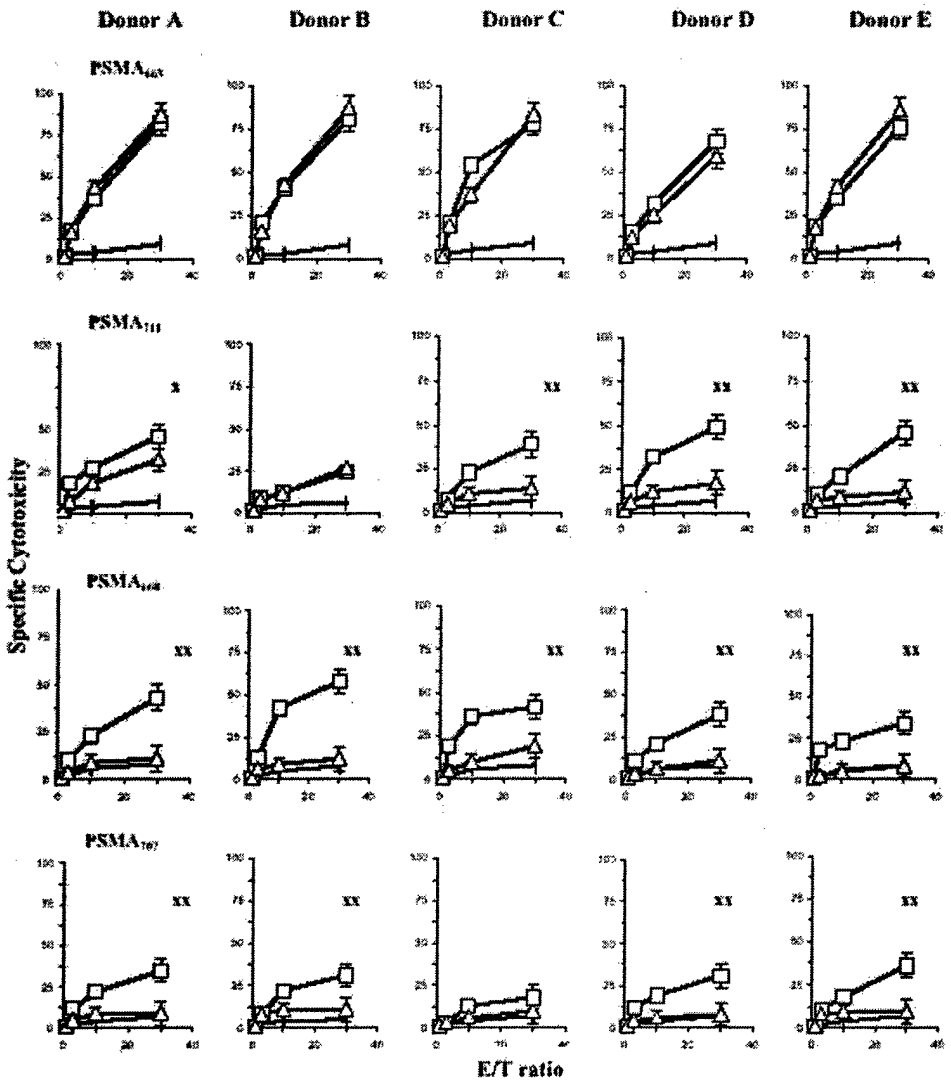

Fig.5. Induction of CTL responses with autologous dendritic cells that have been transfected with the extracellular domain of PSMA. Peripheral blood mononuclear cells that had been depleted of monocytes were primed with autologous DCs transfected with either sPSMA (triangles), tPSMA (squares) or control (mock, empty) plasmids (vertical bar). Responding cultures were then boosted with monocytes pulsed with one of four HLA-A2 restricted PSMA-derived peptides (table 1) and their cytotoxicity tested against T2 cells pulsed with the same peptide that was used during boosting, or with a control (influenza derived) peptide. Data points for the control peptide are not shown but are identical to those obtained with empty plasmid-transfected DCs. Each point represents the mean and SD of triplicate experiments. Significant differences ($p<0.01$) in cytotoxicity against specific and control targets at the 30:1 E/T ratio are indicated (**xx**).

second higher-affinity receptor for B7, termed CTLA-4.[29] The latter inhibits T cell activation at instances of weak T cell receptor engagement[30] and we speculated that we could enhance T cell stimulation to existing subdominant epitopes if we inhibited CTLA-4 signaling with anti-CTLA-4 Fab fragments. Indeed, CTLA-4 inhibition during sPSMA DC-priming led to stimulation of T cells reactive to sub-dominant epitopes. We interpret this as evidence that sub-dominant epitopes

are generated in DC transfected with the sPSMA plasmid but the response can be detected only after CTLA-4 inhibition (fig.6).

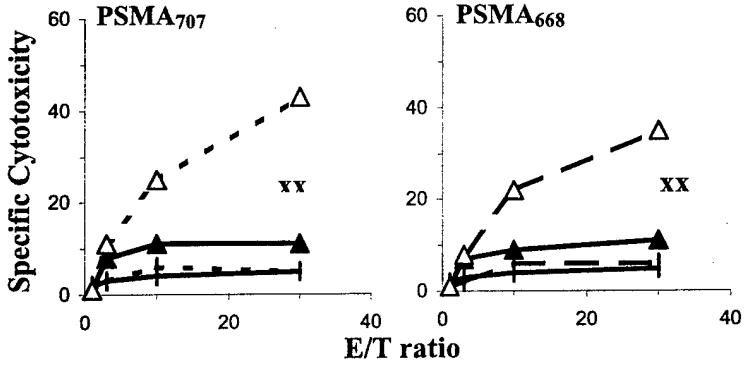

Fig. 6. HLA A2 (+) peripheral blood mononuclear cells that had been depleted of monocytes were primed with autologous dendritic cells transfected with either sPSMA plasmid (triangles) or control (mock, empty) plasmid (diamonds) in the presence (open symbols) or absence (closed symbols) of blocking anti-CD152 Fab fragments. The Fab fragments (0.02 mg/ml) were added at the initiation of the culture. Three days later, and then 2 more times at weekly intervals, the responding cultures were boosted with monocytes pulsed with one of two HLA-A2 restricted, PSMA-derived peptides ($PSMA_{707}$ or $PSMA_{668}$). Cytotoxicity was tested against T2 cells pulsed with the same peptide that was used during boosting, or with a control (influenza) peptide. Data points for the control peptide are not shown but are identical to those obtained with empty plasmid-transfected DCs. Results from 3 independent experiments are shown. Significant differences ($p<0.01$) in cytotoxicity against specific and control targets at the 30:1 E/T ratio are indicated (xx).

Since CTLA-4 was not expressed in resting T cells, we determined the time points during the 72-hour priming phase that the inhibitory control by CTLA-4 became apparent. The anti-CD152 antibody Fab fragments (20 μg/ml) were added either at the beginning, or 6, 10 or 18 hours after the start of the culture. CTLA-4 inhibition was effective when performed early (0 or 6 hours) after initiation of culture (fig.7).

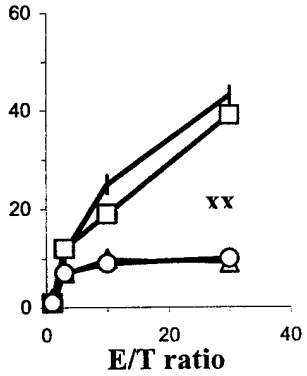

Fig. 7. HLA A2 (+) peripheral blood mononuclear cells depleted of monocytes were primed in the presence of blocking anti-CD152 Fab fragments with autologous dendritic cells that had been transfected with sPSMA plasmid. The Fab fragments (0.02 mg/ml) were added either at the beginning (diamonds), or 6 (squares), 10 (triangles) or 18 (circles) hours after initiation of culture. Lymphocytes were then cultured for 3 days, then washed, resuspended in IL-2 and IL-7 AIM-V medium and boosted twice at 1-week interval with autologous $PSMA_{707}$-pulsed monocytes. Cytotoxicity was tested against T2 cells pulsed with the same peptide. Results from 3 independent experiments are shown. Significant differences ($p<0.01$) in cytotoxicity against specific and control targets at the 30:1 E/T ratio are indicated (xx)

Repeated boosting with transfected DCs restricts the response towards one immunodominant epitope

Maturation of potent CTL effectors requires repetitive boosting with target antigen. To determine the effect of a prime/boost vaccination strategy on the clonality of the T cell response, tPSMA DCs-primed cultures, known to contain CTLs to sub-dominant epitopes (fig.5), were boosted with transfected or peptide pulsed dendritic cells or monocytes, and their cytotoxicity was tested against PSMA-peptide pulsed T2 target cells. Boosting with either antigen presenting cells that express multiple PSMA-derived epitopes (transfected DCs, or DCs or monocytes pulsed with multiple peptides) restricts the immune response towards one immunodominant epitope (table 2). A subdominant T cell response could only be preserved if boosting is performed with an APC pulsed with the particular subdominant epitope.

Table 2. Boosting with dendritic cells that express multiple antigenic epitopes restricts the response to an immunodominant epitope

Prime/Boost Strategy*	**Number of Patients Developing CTL Activity against PSMA Peptide-Pulsed T2 cells**			
	A_{663}	A_{711}	A_{668}	A_{707}
Prime: tPSMA DCs. 2 Boosts tPSMA DCs	5 of 5	1 of 5	0 of 5	0 of 5
Prime: tPSMA DCs. 2 Boosts sPSMA DCs	5 of 5	1 of 5	0 of 5	0 of 5
Prime: tPSMA DCs. 2 Boosts 1P DCs	5 of 5	4 of 5	5 of 5	4 of 5
Prime: tPSMA DCs. 2 Boosts 4P Mos	5 of 5	0 of 5	0 of 5	0 of 5
Prime: tPSMA DCs. 2 Boosts 1P Mos	5 of 5	4 of 5	5 of 5	4 of 5

* 1P – pulsed with single peptide, 4P – pulsed with all four peptides, Mos- monocytes

Anti-tumor reactivity of PSMA-specific CTLs

The ability of the PSMA-reactive T cell clones to recognize tumor cells that express PSMA was tested in an in vitro cytotoxicity assay. In all 5 donors, only two of the peptide-specific CTL clones ($PSMA_{663}$ and $PSMA_{711}$) were cytotoxic against non-modified LNCaP cells (fig.8).

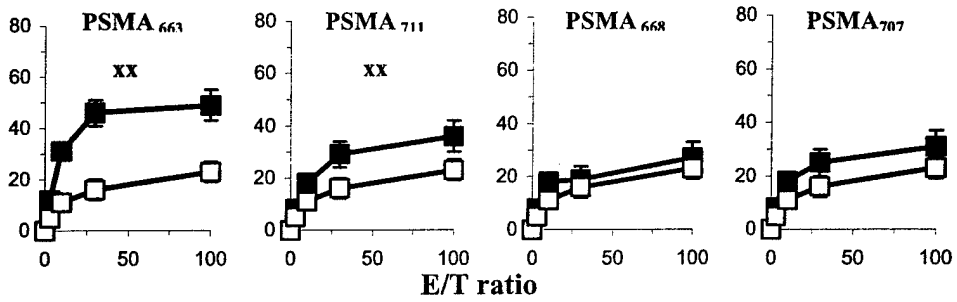

Fig. 8. Anti-tumor reactivity of PSMA peptide-specific CTLs. Peptide-reactive CTL lines shown in fig. 5 were tested for their ability to recognize and kill prostate tumor cells. LNCaP cells (filled symbols) or Malme-3M melanoma cells were used as targets. Each point represents the mean and SD of triplicate experiments. Significant differences ($p<0.01$) in cytotoxicity against specific and control targets at the 30:1 E/T ratio are indicated (xx). A representative result from five tested donors is shown.

DISCUSSION

Despite being effective in breaking tolerance to a self-antigen or tumor-associated antigen, anti-tumor vaccination in its current mode of application leads to a narrow, directed to few of the potential epitopes response. Immunodominance is a natural control mechanism that ensures the tight specificity of the immune reaction and prevents untoward autoimmunity.[19] Immunodominance, however, presents a problem in vaccinology since loss of an MHC haplotype that participates in the conformation of the T cell antigen, or point mutation in the recognized sequence, would result in ineffective immune surveillance.[16,17] We have explored possibilities to extend the target epitope specificity of the immune response and the following study was designed to test the ability of dendritic cells transfected with different plasmid constructs to prime and boost cytotoxic T cell responses.

A critical requirement for T cell activation is the engagement of TCRs with major histocompatibility complex molecules presenting antigenic peptides on the surface of an antigen-presenting cell.[31] Efficient priming of multi-specific CTL responses is limited by the poor immunogenicity of subdominant MHC class I-binding epitopes.[19] T cells binding with high affinity to the antigen compete with low affinity T cells and inhibit their interaction with the same antigen-presenting cell by down-modulation of peptide-MHC complexes on the APC.[32] Inhibition of responses to sub-dominant epitopes, therefore, could be partially overcome by simply increasing the amount of antigen present on the APCs.[33-35] We reasoned that, since proteasomal degradation is the main source of antigenic fragments destined for MHC class I presentation,[14] purposeful cytosolic retention of newly synthesized tumor-associated antigens in genetically manipulated antigen presenting cells may enhance their proteasomal degradation and thus increase the quantity of such fragments. The transport from the cytosol into the endoplasmic reticulum (ER) is an important step in the biogenesis of many proteins, including secretory proteins and proteins of the plasma membrane. It is triggered by a signal sequence, which is normally located at the amino terminus of those polypeptides. For many membrane proteins, the signal sequence is identical with the first membrane anchor (signal-anchor sequence).[36] Since PSMA is a type II, bitopic integral membrane protein, we speculated that elimination of the sequence that encodes for its transmembrane domain will lead to expression of a product that will not be translocated to the ER, but will be degraded by the proteasomes, presumably to

peptides that will access the class I presentation pathway. Indeed, the product expressed following transfection of COS-1 cells with such "truncated" construct is not glycosylated, but is retained in the cytosol and rapidly degraded by the proteasomes. This leads to generation of PSMA peptides that are expressed via the class I MHC presentation pathway at density sufficient to prime CTLs to all four PSMA epitopes tested.

Contrary to that, dendritic cells transfected with DNA encoding for a product, which is translocated to the ER, then glycosylated and secreted, do not prime against subdominant epitopes (fig.5). Although all four PSMA epitopes are generated, T cells reactive to the subdominant ones could only be primed if CTLA-4 signaling is inhibited (fig.6). Since high-affinity receptor T cells down-modulate peptide-MHC complexes from the APC membrane,[37] increased production of peptides after tPSMA transfection favors sub-dominant CTL priming. Stimulation to subdominant MHC-peptide complexes that are expressed at lower density on the APC membrane following sPSMA transfection, however, requires CTLA-4 inhibition. A homologue of CD28, CTLA-4 also binds to the B-7 family members [38,39] but inhibits T cell activation.[40] Mice lacking CTLA-4 reveal a striking phenotype of polyclonal T cell activation and tissue infiltration which results in death by 3-4 weeks of age, indicating a powerful regulatory role for CTLA-4.[41,42]

Our finding that priming to subdominant responses is enhanced by CTLA-4 inhibition may seem contradictory to the model proposed by others.[43] In that model CTLA-4 signaling and not inhibition allows for a greater diversity in the T cell response. Our results, however, support the model originally proposed by Manzotti et al..[30] The following operational mechanisms are, therefore, possible, but their clarification requires additional experimentation:

First, the intensity of TCR stimulation seems to be important with weak signals being prompt to inhibition.[30] In other words, weak signals being overwhelmed by inhibition through CTLA-4 will have no chance to prime CTL responses.

Second, CTLA-4 may act as a non-signaling"decoy" receptor reducing the available ligand for CD28 costimulation.[44,45]

There is also a third possibility [46,47] that a small number of CTLA-4-expressing and -stimulated T cells exerts a suppressive or regulatory effect on other T cells. These cells appear to be similar if not identical to T regulatory cells.[48-50]

Experiments are currently in progress to validate any of these hypotheses.

Priming with tPSMA DCs leads to polyclonal CTL stimulation, but boosting with APCs that express both dominant and sub-dominant epitopes narrows the immune response to the dominant ones. Research from other groups has gained similar results.[51-55] In all these instances, boosting with polyepitope constructs has resulted in failure to expand polyepitope CTLs. A likely explanation is that competition between T cells for antigen on individual APC leads to obscuring of responses to sub-dominant epitopes when both the dominant and subdominant epitopes are present on the same APC.[55,56] New vaccines or new strategies employing modulation of co-stimulatory networks during boosting may need to be developed if the polyepitope response against a target is to be maintained.

Finally, not all PSMA-peptide specific CTL clones exhibited tumor cell killing (fig.8). A likely explanation for the lack of cytotoxicity is the downregulation of MHC - unlike other tumor cells, LNCaP cells express low levels of MHC class I molecules.[57] However, neither the level of class I MHC expression, nor the percentage of killing, was influenced by pre-treatment of the target cells with γ-IFN (data not shown). Alternatively, these subdominant epitopes may not be

generated at sufficient quantity by LNCaP cells and further experimentation is necessary to test this possibility.

In conclusion, we have shown that:

1. Dendritic cells transfected with plasmid DNA can successfully prime CTL responses in vitro.
2. Dendritic cells transfected with a construct whose product is retained in the cytosol and degraded in the proteasome (tPSMA), prime to both dominant and subdominant epitopes. In contrast, sPSMA DCs prime to dominant epitopes only.
3. CTLA-4 inhibition during priming in vitro enhances priming to sub-dominant epitopes generated following transfection of DCs with sPSMA.
4. In vitro boosting with APCs that express both dominant and sub-dominant epitopes narrows the immune response to the dominant ones.

If confirmed in animal studies, these results will pose important questions on the design of vaccines and methods for re-immunization if a polyepitope response is to be maintained.

REFERENCES

1. Mincheff M, Tchakarov S, Zoubak S, et al. Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: a phase I/II clinical trial. *Eur Urol.* 2000;38(2):208-217.
2. Mincheff M, Altankova I, Zoubak S, et al. In vivo transfection and/or cross-priming of dendritic cells following DNA and adenoviral immunizations for immunotherapy of cancer--changes in peripheral mononuclear subsets and intracellular IL-4 and IFN-gamma lymphokine profile. *Crit Rev Oncol Hematol.* 2001;39(1-2):125-132.
3. Weber LW, Bowne WB, Wolchok JD, et al. Tumor immunity and autoimmunity induced by immunization with homologous DNA. *J Clin Invest.* 1998;102(6):1258-1264.
4. Israeli RS, Powell CT, Fair WR, et al. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.* 1993;53(2):227-230.
5. Wright GL, Jr., Grob BM, Haley C, et al. Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. *Urology.* 1996;48(2):326-334.
6. Chang SS, Gaudin PB, Reuter VE, et al. Prostate-specific membrane antigen: present and future applications. *Urology.* 2000;55(5):622-629.
7. Chang SS, Gaudin PB, Reuter VE, et al. Prostate-Specific Membrane Antigen: Much More Than a Prostate Cancer Marker. *Mol Urol.* 1999;3(3):313-320.
8. Heston WD. [Significance of prostate-specific membrane antigen (PSMA). A neurocarboxypeptidase and membrane folate hydrolase]. *Urologe A.* 1996;35(5):400-407.
9. Silver DA, Pellicer I, Fair WR, et al. Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin Cancer Res.* 1997;3(1):81-85.
10. Shneider BL, Thevananther S, Moyer MS, et al. Cloning and characterization of a novel peptidase from rat and human ileum. *J Biol Chem.* 1997;272(49):31006-31015.
11. Halsted CH, Ling E, Luthi-Carter R, et al. Folylpoly-gamma -glutamate carboxypeptidase from pig jejunum. Molecular characterization and relation to glutamate carboxypeptidase II. *J Biol Chem.* 2000;275(39):30746.
12. Chang SS, O'Keefe DS, Bacich DJ, et al. Prostate-specific membrane antigen is produced in tumor-associated neovasculature. *Clin Cancer Res.* 1999;5(10):2674-2681.
13. Sprent J, Schaefer M. Antigen-presenting cells for unprimed T cells. *Immunol Today.* 1989;10(1):17-23.
14. Bennink JR, Anderson R, Bacik I, et al. Antigen processing: where tumor-specific T-cell responses begin. *J Immunother.* 1993;14(3):202-208.
15. Restifo NP, Kawakami Y, Marincola F, et al. Molecular mechanisms used by tumors to escape immune recognition: immunogenetherapy and the cell biology of major histocompatibility complex class I. *J Immunother.* 1993;14(3):182-190.
16. Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol.* 2002;3(11):991-998.
17. Schreiber H, Wu TH, Nachman J, et al. Immunodominance and tumor escape. *Semin Cancer Biol.* 2002;12(1):25-31.
18. Ikeda H, Old LJ, Schreiber RD. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev.* 2002;13(2):95-109.
19. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu Rev Immunol.* 1999;17:51-88.
20. Pinto JT, Suffoletto BP, Berzin TM, et al. Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin Cancer Res.* 1996;2(9):1445-1451.

21. Rawlings ND, Barrett AJ. Structure of membrane glutamate carboxypeptidase. *Biochim Biophys Acta*. 1997;1339(2):247-252.
22. Holmes EH, Greene TG, Tino WT, et al. Analysis of glycosylation of prostate-specific membrane antigen derived from LNCaP cells, prostatic carcinoma tumors, and serum from prostate cancer patients. *Prostate Suppl*. 1996;7:25-29.
23. Barinka C, Rinnova M, Sacha P, et al. Substrate specificity, inhibition and enzymological analysis of recombinant human glutamate carboxypeptidase II. *J Neurochem*. 2002;80(3):477-487.
24. DeMars R, Chang CC, Shaw S, et al. Homozygous deletions that simultaneously eliminate expressions of class I and class II antigens of EBV-transformed B-lymphoblastoid cells. I. Reduced proliferative responses of autologous and allogeneic T cells to mutant cells that have decreased expression of class II antigens. *Hum Immunol*. 1984;11(2):77-97.
25. Matzinger P. The JAM test. A simple assay for DNA fragmentation and cell death. *J Immunol Methods*. 1991;145(1-2):185-192.
26. Parker KC, Bednarek MA, Hull LK, et al. Sequence motifs important for peptide binding to the human MHC class I molecule, HLA-A2. *J Immunol*. 1992;149(11):3580-3587.
27. Kaye PM. Costimulation and the regulation of antimicrobial immunity. *Immunol Today*. 1995;16(9):423-427.
28. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol*. 1996;14:233-258.
29. Walunas TL, Lenschow DJ, Bakker CY, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity*. 1994;1(5):405-413.
30. Manzotti CN, Tipping H, Perry LC, et al. Inhibition of human T cell proliferation by CTLA-4 utilizes CD80 and requires CD25+ regulatory T cells. *Eur J Immunol*. 2002;32(10):2888-2896.
31. Davis MM, Chien YH, Bjorkman PJ, et al. A possible basis for major histocompatibility complex-restricted T-cell recognition. *Philos Trans R Soc Lond B Biol Sci*. 1989;323(1217):521-524.
32. Kedl RM, Schaefer BC, Kappler JW, et al. T cells down-modulate peptide-MHC complexes on APCs in vivo. *Nat Immunol*. 2002;3(1):27-32.
33. Mitchison NA. Specialization, tolerance, memory, competition, latency, and strife among T cells. *Annu Rev Immunol*. 1992;10:1-12.
34. Lightstone E, Marvel J, Mitchison A. Memory in helper T cells of minor histocompatibility antigens, revealed in vivo by alloimmunizations in combination with Thy-1 antigen. *Eur J Immunol*. 1992;22(1):115-122.
35. Clark EA, Lake P, Favila-Castillo L. Modulation of Thy-1 alloantibody responses: donor cell-associated H-2 inhibition and augmentation without recipient Ir gene control. *J Immunol*. 1981;127(5):2135-2140.
36. Rapoport TA, Rolls MM, Jungnickel B. Approaching the mechanism of protein transport across the ER membrane. *Curr Opin Cell Biol*. 1996;8(4):499-504.
37. Kedl RM, Rees WA, Hildeman DA, et al. T cells compete for access to antigen-bearing antigen-presenting cells. *J Exp Med*. 2000;192(8):1105-1113.
38. Greene JL, Leytze GM, Emswiler J, et al. Covalent dimerization of CD28/CTLA-4 and oligomerization of CD80/CD86 regulate T cell costimulatory interactions. *J Biol Chem*. 1996;271(43):26762-26771.
39. van der Merwe PA, Bodian DL, Daenke S, et al. CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J Exp Med*. 1997;185(3):393-403.

40. Thompson CB, Allison JP. The emerging role of CTLA-4 as an immune attenuator. *Immunity*. 1997;7(4):445-450.
41. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science*. 1995;270(5238):985-988.
42. Tivol EA, Borriello F, Schweitzer AN, et al. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity*. 1995;3(5):541-547.
43. Egen JG, Allison JP. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity*. 2002;16(1):23-35.
44. Masteller EL, Chuang E, Mullen AC, et al. Structural analysis of CTLA-4 function in vivo. *J Immunol*. 2000;164(10):5319-5327.
45. Doyle AM, Mullen AC, Villarino AV, et al. Induction of cytotoxic T lymphocyte antigen 4 (CTLA-4) restricts clonal expansion of helper T cells. *J Exp Med*. 2001;194(7):893-902.
46. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med*. 2000;192(2):295-302.
47. Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med*. 2000;192(2):303-310.
48. Shevach EM. Regulatory T cells in autoimmmunity*. *Annu Rev Immunol*. 2000;18:423-449.
49. Groux H, Powrie F. Regulatory T cells and inflammatory bowel disease. *Immunol Today*. 1999;20(10):442-445.
50. Sakaguchi S. Regulatory T cells: mediating compromises between host and parasite. *Nat Immunol*. 2003;4(1):10-11.
51. Smith SG, Patel PM, Porte J, et al. Human dendritic cells genetically engineered to express a melanoma polyepitope DNA vaccine induce multiple cytotoxic T-cell responses. *Clin Cancer Res*. 2001;7(12):4253-4261.
52. Mateo L, Gardner J, Chen Q, et al. An HLA-A2 polyepitope vaccine for melanoma immunotherapy. *J Immunol*. 1999;163(7):4058-4063.
53. Firat H, Garcia-Pons F, Tourdot S, et al. H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies. *Eur J Immunol*. 1999;29(10):3112-3121.
54. Loirat D, Lemonnier FA, Michel ML. Multiepitopic HLA-A*0201-restricted immune response against hepatitis B surface antigen after DNA-based immunization. *J Immunol*. 2000;165(8):4748-4755.
55. Palmowski MJ, Choi EM, Hermans IF, et al. Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. *J Immunol*. 2002;168(9):4391-4398.
56. Kedl RM, Kappler JW, Marrack P. Epitope dominance, competition and T cell affinity maturation. *Curr Opin Immunol*. 2003;15(1):120-127.
57. Lu J, Celis E. Recognition of prostate tumor cells by cytotoxic T lymphocytes specific for prostate-specific membrane antigen. *Cancer Res*. 2002;62(20):5807-5812.